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Acr3p is a plasma membrane antiporter that catalyzes As(III)/H^+ and Sb(III)/H^+ exchange in *Saccharomyces cerevisiae*

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ABSTRACT

Resistance to arsenical compounds in *Saccharomyces cerevisiae* as well as in a growing number of prokaryotes and eukaryotes is mediated by members of the Acr3 family of transporters. In yeast cells, it has been clearly shown that Acr3p is localized to the plasma membrane and facilitates efflux of trivalent arsenic and antimony. However, until now, the energy dependence and kinetic properties of Acr3 proteins remained uncharacterized. In this work, we show that arsenite and antimonite uptake into everted membrane vesicles via the yeast Acr3 transporter is coupled to the electrochemical potential gradient of protons generated by the plasma membrane H^+-translocating P-type ATPase. These results strongly indicate that Acr3p acts as a metalloid/H^+ antiporter. Two differential kinetic assays revealed that Acr3p-mediated arsenite/H^+ and antimonite/H^+ exchange demonstrates Michaelis–Menten-type saturation kinetics characterized by a maximum flux for permeating metalloids. The approximate K_m values for arsenite and antimonite transport were the same, suggesting that Acr3p exhibits similar low affinity for both metalloids. Nevertheless, the maximal velocity of the transport at saturation concentrations of metalloids was approximately 3 times higher for arsenite than for antimonite. These findings may explain a predominant role of Acr3p in conferring arsenite tolerance in *S. cerevisiae*.

1. Introduction

Arsenic and antimony are naturally occurring toxic metalloids that are readily taken up by organisms when present in excess in soil and water [1,2]. All organisms developed complex mechanisms to avoid toxic levels of arsenite (As(III)) and antimonite (Sb(III)) within the cells, among which metalloid-specific membrane transport proteins play a crucial role [3–5].

In the yeast *Saccharomyces cerevisiae*, tolerance to arsenicals is mediated by the cluster of 3 genes *ACR1*, *ACR2*, and *ACR3*, isolated on a multicopy plasmid conferring 10-fold increase in As(III) resistance in wild-type cells [6]. Acr1p (Yap8p) is a transcription factor which induces expression of *ACR2* and *ACR3* genes from a common promoter region in response to As(III), arsenate (As(V)), and Sb(III) [6–9]. Acr2p is an arsenate reductase which converts As(V) to As(III) [10,11], while Acr3p constitutes the main pathway for As(III) extrusion [12–14]. In addition, As(III) and Sb(III) in the form of glutathione conjugates are accumulated in the vacuole by the ABC transporter Ycf1p as a second pathway of metalloid detoxification in yeast [13].

The yeast Acr3p is the first functionally characterized [12,13] and prototype member of the arsenical compound resistance-3 (Acr3) family of transporters which belongs to the bile/arsenite/riboflavin transporter (BART) superfamily [15], although the first *ACR3* gene sequence has been identified in the bacterial genome of *Bacillus subtilis* [16,17]. Acr3 homologs are ubiquitously present in prokaryotes and fungi. Recently, several new members of Acr3 family have been found in the plant genomes of moss, lycophytes, ferns, and gymnosperms [18,19]. Acr3 transporters are characterized by the predicted or confirmed 10-transmembrane span topology [12,18–20] and specificity towards As(III) [12,13,17,18]. However, Acr3 homolog from *Synechocystis* sp. confers resistance to both As(III) and Sb(III) [21], while the *S. cerevisiae* Acr3 has been recently shown to mediate Sb(III) efflux and moderate level of tolerance to this metalloid [14]. Thus, at least in some organisms, Acr3 has a dual As(III)/Sb(III) specificity. On the other hand, the *Shewanella oneidensis* Acr3 confers resistance to As(V) and does not bind As(III), suggesting that As(V) is the substrate of this transporter [22]. Interestingly, the *Mycobacterium tuberculosis Rv2643* gene encoding for an unusual Acr3 protein fused to an arsenate reductase ArsC domain also mediates As(V) resistance when expressed in the arsenic-sensitive mutant of *S. cerevisiae* but probably couples As(V) reduction with immediate As(III) efflux [23]. In microorganisms, Acr3 is localized to the plasma membrane to mediate As(III) export out of the cell [12–14,17,18]. However, Acr3 from the arsenic hyperaccumulating fern *Pteris vittata* is present at the vacuolar membrane in gametophytes which suggests that in multicellular organisms Acr3 may mediate sequestration of As(III) into the vacuole [19].

The role of Acr3 proteins in catalyzing As(III) transport is well-established based on several genetic, physiological, and *in vivo*

Abbreviations: As(III), arsenite; As(V), arsenate; Sb(III), antimonite; GFP, green fluorescent protein

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transport studies [12–14,17–19]. Until now, mechanism of metalloid transport via Acr3 has not been studied yet. Here, we demonstrate that the *S. cerevisiae* Acr3p acts as an As(III)/H^+ and Sb(III)/H^+ antiporter at the plasma membrane. Interestingly, although Acr3p showed similar affinity for both As(III) and Sb(III), As(III) was transported much faster than Sb(III) which is in good agreement with a secondary role of Acr3p in mediating Sb(III) tolerance in yeast as previously suggested [14].

2. Materials and methods

2.1. Yeast strains, plasmids, and growth conditions

The yeast *S. cerevisiae* wild type strain W303-1A (*MAT*a *ura3 leu2 trp1 his3 ade2 can1*) and the *acr3* deletion mutant RW104 (W303-1A, *acr3Δ::kanMX*) [24] bearing either an empty plasmid pUG35 (centromeric *S. cerevisiae/Escherichia coli* shuttle vector for C-terminal gene fusion with GFP, *URA3*, Amp^R) [25] or pACR3-GFP (pUG35 containing *ACR3*-GFP fusion gene under the control of the native promoter) [14] were used in this study. Yeast transformants were grown in selective synthetic minimal medium at 28 °C [26]. Yeast transformations were done by the lithium acetate procedure [27]. Growth tests in the presence of As(III) (sodium arsenite) and Sb(III) (potassium antimonyl tartrate) were carried out as previously described [24].

2.2. Isolation of plasma membrane vesicles

Cells from two liters of the *S. cerevisiae acr3*Δ + pACR3-GFP or *acr3*Δ + pUG35 culture grown to OD_{600} of 0.7–0.9 and treated with 1 mM As(III) for 4 h to induce Acr3p expression followed by extensive washing were used for isolation of plasma membranes. Inside-out plasma membrane vesicles were prepared using a dextran/polyethylene glycol two-phase partitioning method as described previously [28]. Freshly prepared plasma membranes were immediately used for ATPase and transport assays.

2.3. ATPase assays

Several assays of ATP hydrolysis were performed to determine the purity and orientation of the plasma membrane vesicle fraction. The standard reaction mixture of contained 10 mM MES–Tris (pH 6.0), 330 mM sucrose, 140 mM KCl, 4 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 2 mM ATP, and 25 μg of membrane protein. To assess the purity of the vesicle preparation, the standard incubation condition was compared with incubation in a medium containing 0.2 mM sodium orthovanadate, 1 mM sodium azide, 0.1 mM ammonium molybdate, or 50 mM potassium nitrate. The assay was conducted at 30 °C for 30 min. The level of inorganic phosphate was measured according to Ames [29]. Determination of the orientation of the plasma membrane vesicles was based on the latency of the plasma membrane H^+-ATPase, Pma1p. In this assay, incubation with 0.02% Triton X-100 or 0.02% Brij 58 was compared with a standard incubation as described above.

2.4. Transport assays

Formation of the pH gradient generated by the plasma membrane H^+-ATPase was monitored by the quenching of acridine orange absorbance [30,31]. The reaction mixture contained 10 mM MES–Tris (pH 6.0), 330 mM sucrose, 140 mM KCl, 4 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 5 μM acridine orange, and 50 μg membrane protein. Immediately, after addition of 2 mM ATP into the medium (final pH 7.5), generation of the proton gradient was observed as a quenching of acridine orange absorbance using a spectrophotometer (Beckman DU® 640) at 495 nm. In preliminary experiments, the effect of 0.2 mM sodium orthovanadate, 5 mM NH_4Cl, or 0.4 μg ml^{-1} gramicidin D on the maintenance of the proton gradient of isolated plasma membranes was tested to confirm whether the vesicles were functionally sealed. To examine the effect of metalloids on ΔpH, different concentrations of As(III) and Sb(III) were added to the plasma membrane vesicles with the established pH gradient followed by inhibition of H^+-ATPase with the addition of 0.2 mM orthovanadate and changes of the acridine orange absorbance were monitored for a period of 3 min. In control experiments, metalloids were added to the membranes without previous generation of proton gradient.

Direct measurements of As(III) and Sb(III) uptake into everted membrane vesicles were determined using direct filtration assay and atomic absorption spectrometry. Fifty micrograms of membrane protein were introduced into the reaction mixture containing 10 mM MES–Tris (pH 6.0), 330 mM sucrose, 1 mM DTT, 140 mM KCl, and 4 mM $MgCl_2$. The formation of proton gradient across the membranes was initiated by the addition of 2 mM ATP and stopped after 3 min with 0.2 mM sodium orthovanadate. Subsequently, different concentrations of metalloids were added to the membranes for a period of 3 min. Then, the samples were filtered through nitrocellulose (0.45 μm pore size; Bio-Rad) and washed with 5 ml of the same buffer. The membranes bound to the filters were washed with 2 ml of 3 M HCl to rinse out As(III) or Sb(III). The metalloid content was determined using a flame atomic absorption spectrometer (3300; PerkinElmer). The corrections for unspecific binding of metalloids to the outer side of the membranes or filters were made by the simultaneous incubation of vesicles in the conditions without pH gradient, when 0.2 mM sodium orthovanadate was introduced into reaction media along with ATP.

2.5. Immunoblotting

The plasma membrane-enriched protein fractions were resolved on 10% SDS–PAGE gel, blotted onto nitrocellulose filters (Bio-Rad) and probed with the anti-GFP antibody (Roche) at a dilution of 1:4000 to detect the presence of Acr3-GFP and the anti-Pma1 antibody (kindly provided by M. Ghislain, Université Catholique de Louvain, Belgium) at a dilution of 1:15,000 for quality control of the membrane-enriched fraction. Protein content was quantified using the Bio-Rad protein assay reagent and bovine serum albumin as a standard.

3. Results

Acr3p confers resistance to toxic metalloids by mediating extrusion of As(III) and Sb(III) out of yeast cells [12–14]. However, mechanism and energy dependence of metalloid efflux via Acr3 protein has never been investigated in any organism. Based on the fact that Acr3p does not contain ATP-binding motif, it was speculated that Acr3p couples metalloid transport to the proton-motive force and may function as either a $As(OH)_3/H^+$ antiporter or a uniporter catalyzing movement of $As(OH)_2O^-$ anion [13,18].

To elucidate the mechanism of Acr3p-mediated metalloid transport in the yeast *S. cerevisiae*, we prepared everted plasma membrane vesicles from the *acr3*Δ mutant containing an empty vector pUG35 for control or expressing Acr3p-GFP fusion protein from the pACR3-GFP plasmid. The results of ATP hydrolysis assays clearly showed that the separation of the plasma membranes on a dextran/polyethylene glycol two-phase system yielded in the highly purified (90%) plasma membrane vesicles (Fig. 1A). The hydrolytic activities of the marker enzymes specific for other membranes (vacuolar V-ATPase and mitochondrial ATPase) and cytosol (alkaline phosphatase) were significantly low but detectable; therefore, azide, nitrate, and molybdate were introduced into further assays of metalloid transport to inhibit these other activities. On the contrary, the marker plasma membrane H^+-ATPase Pma1p displayed considerably high hydrolytic and transport activities in the same purified membrane fraction (Fig. 1A and B). Isolated plasma membrane vesicles had the inside-out orientation since ATPase activity was not dependent on the addition of detergents (Triton X-100 or Brij 58) (data not shown). The preliminary assay of proton transport

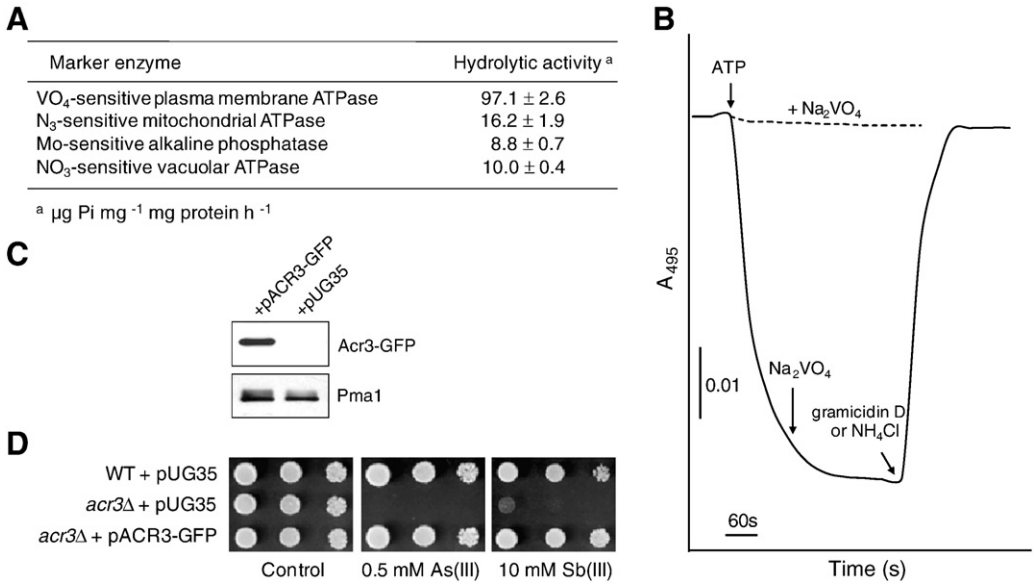

Fig. 1. Properties of the plasma membrane vesicles isolated from yeast spheroplasts expressing Acr3p. (A) Marker enzyme activities in isolated membrane vesicles. Enzyme activity was calculated from a difference between the amount of inorganic phosphate produced in the presence or absence of specific inhibitors of various ATPases. The values represent the SD of three replications. (B) ATP-energized H^+ transport activity was measured as described in Materials and methods. Two micromolar ATP was added at the first arrow, which initiated a rapid acridine orange fluorescence decrease indicating acidification of vesicles (solid line). The addition of 0.2 mM sodium orthovanadate (Na_2VO_4) along with ATP completely prevented proton gradient generation (dashed line). At the second arrow, 0.2 mM sodium orthovanadate was added to inhibit H^+-ATPase and maintain a steady-state acidic-inside pH gradient. At the third arrow, 5 μM gramicidin D or 10 mM NH_4Cl was added to collapse the ΔpH gradient across the plasma membrane. Presented values are representative for the results obtained in three to four independent experiments with each experiment done in triplicate. (C) Immunodetection of Acr3p-GFP and Pma1p in the membrane vesicles prepared from the *acr3*Δ + pACR3-GFP or *acr3*Δ + pUG35 (empty vector) yeast cells. Blots were probed with the anti-GFP antibody to detect Acr3p-GFP and the anti-Pma1 antibody as a loading and plasma membrane quality control. (D) The Acr3-GFP fusion protein is functional. Cultures of the indicated yeast transformants were spotted on selective minimal medium plates in the presence or absence of metalloids. Plates were photographed after 3 days at 28 °C.

confirmed orientation and purity of the plasma membranes and showed that the vesicles were tightly sealed (Fig. 1B). Namely, the formation of proton gradient across the membranes was observed only when ATP was present in the media and both gramicidin D and NH_4Cl caused the instantaneous dissipation of ΔpH (Fig. 1B). The ATP-dependent ΔpH generation was catalyzed solely by Pma1p since orthovanadate, the specific inhibitor of plasma membrane proton pump, completely inhibited proton transport across membranes (Fig. 1B). Western blotting confirmed the plasma membrane localization of Acr3p-GFP in the *acr3*Δ strain harboring the pACR3-GFP plasmid as well as the lack of the transporter in the plasma membranes isolated from *acr3*Δ mutant harboring the empty vector pUG35 (Fig. 1C). In addition, we showed that expression of Acr3p-GFP fully complements metalloid sensitivity of *acr3*Δ cells, indicating that the Acr3-GFP fusion protein is functional.

To investigate the effect of As(III) or Sb(III) on ATP-driven acidification of isolated plasma membrane vesicles, we measured changes of acridine orange absorbance, weak base used as a ΔpH-sensitive probe. In preliminary experiments, we checked that As(III) and Sb(III) in concentrations up to 10 mM did not interfere with absorbance of acridine orange in the presence or absence of vesicles and ATP (data not shown). As demonstrated in Fig. 2, in the presence of ATP quenching of acridine orange, absorbance was observed which indicates the formation of ΔpH gradient across the vesicle membranes mediated by H^+-ATPase. Orthovanadate stopped further development of intravesicular acidification whereas the addition of As(III) or Sb(III) to the membranes caused an instantaneous, concentration-dependent dissipation of ΔpH only when Acr3p was present in the membranes (Fig. 2A–C). These results demonstrate that Acr3p displays As(III)/H^+ and Sb(III)/H^+ exchange activity. The Acr3p-dependent metalloid/H^+ antiport exhibited saturation kinetics with the apparent K_m of 2 mM for both metalloids (Fig. 2D). Interestingly, the V_{max} for As(III) was approximately 3 times higher than for Sb(III), suggesting that Acr3p is primarily a low-affinity As(III)/H^+ transporter.

To confirm that the metalloid-induced ΔpH dissipation in the plasma membranes is strictly related to the As(III)/H^+ and Sb(III)/H^+ antiport across everted plasma membranes vesicles, accumulation of As(III) and Sb(III) into the vesicles were directly measured using direct filtration assay and atomic absorption spectrometry. In agreement with the measurements of pH gradient changes (Fig. 2), the contents of As(III) and Sb(III) within the vesicles were dependent on the presence of artificial ΔpH in the membranes and increased with the external concentration of the metalloids showing the saturation kinetics (Table 1). Similarly, the rate of As(III) uptake was approximately 3-fold higher than that of Sb(III) (Table 1). The transport of metalloids into everted plasma membrane vesicles was almost completely inhibited when ATP was introduced into reaction media along with the Pma1p inhibitor, orthovanadate (data not shown). Under these conditions, the content of metalloids measured in the plasma membrane vesicles obtained from Acr3p-expressing yeast was comparable to the amount of As(III) and Sb(III) measured in the plasma membrane vesicles lacking Acr3p and incubated with metalloids and ATP but without orthovanadate. Hence, Pma1p-mediated formation of electrochemical gradient (proton motive force) was required for the activation of Acr3p and ATP was not directly involved in the Acr3p-mediated metalloid translocation across the plasma membrane.

4. Discussion

Here, we showed that the yeast Acr3p is a As(III)/H^+ and Sb(III)/H^+ antiporter which depends on the pH gradient generated by the plasma membrane H^+-ATPase. Interestingly, Acr3p displays similar low affinity for both As(III) and Sb(III) but transports As(III) three times faster than Sb(III). This confirms our recent *in vivo* results showing a dual As(III)/Sb(III) activity of Acr3p but a predominant role of this transporter in conferring resistance to As(III) in *S. cerevisiae* [14]. The *acr3*Δ mutant was 10-fold more sensitive to As(III) but only 1.5-fold more sensitive to Sb(III); however, the *acr3*Δ mutant showed decreased Sb(III) efflux, while overexpression of *ACR3* gene complemented high sensitivity to Sb(III) in the *ycf1*Δ mutant lacking the vacuolar ABC transporter and efficiently extruded Sb(III) out of the cell [14]. We hypothesize that

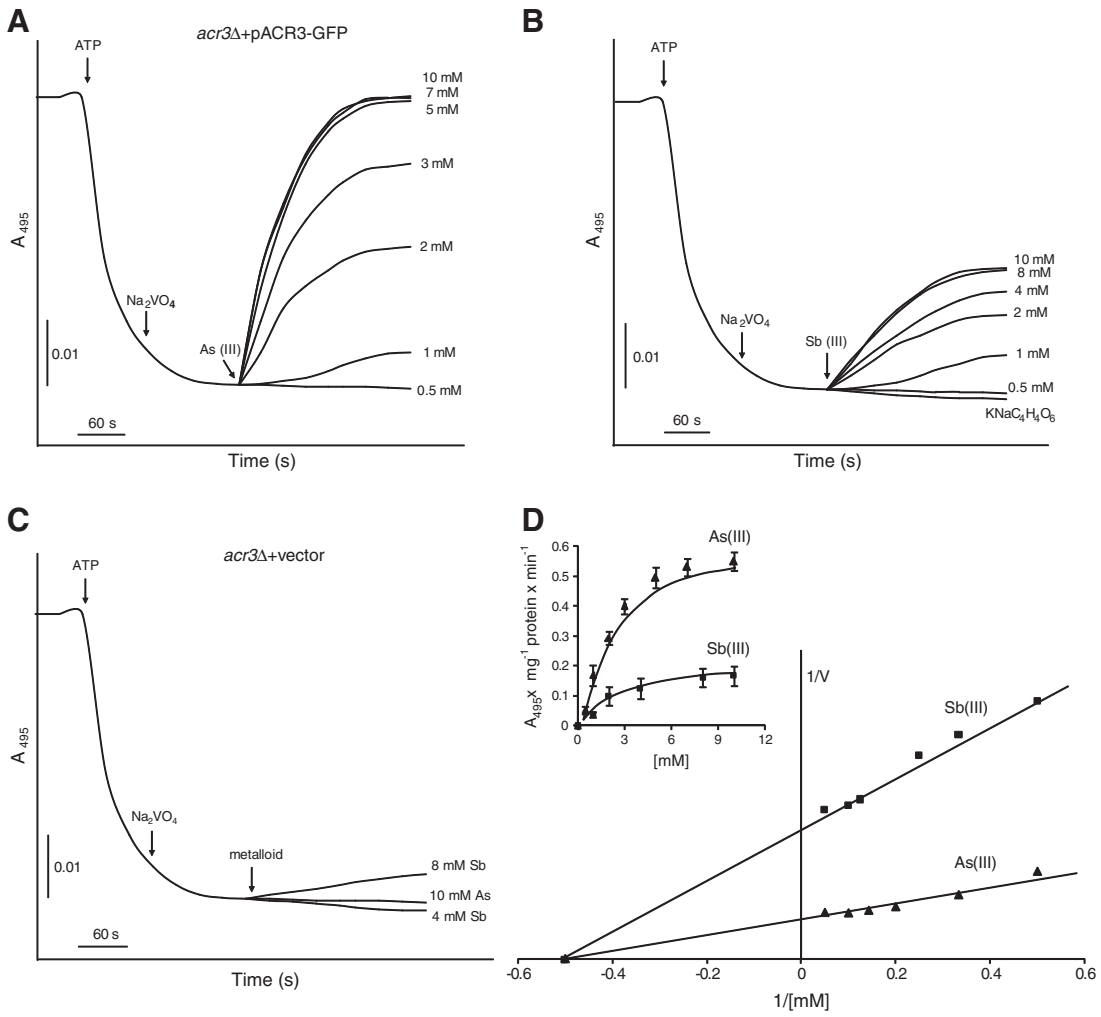

Fig. 2. Metalloid-dependent H^+ transport across everted plasma membrane vesicles. (A–C) Fifty microgram membrane proteins isolated from either *acr3*Δ + pACR3-GFP or *acr3*Δ + pUG35 (empty vector) yeast cells were incubated for 5 min in the reaction media containing acridine orange. ATP was added to the reaction to initiate Pma1p-mediated H^+ influx into the vesicles, monitored as an acridine absorbance drop (first arrow). Next, 0.2 mM sodium orthovanadate was added to inhibit H^+-ATPase and maintain a steady-state acidic-inside pH gradient (second arrow). Different concentrations of sodium arsenite (As(III)) or potassium antimonyl tartrate (Sb(III)) were introduced into the membranes with imposed pH gradient and the subsequent acridine absorbance increase, reflecting the H^+/metalloid antiport activity, was measured in a period of 3 min. Since Sb(III) was supplied as a tartrate salt, a control experiment with 10 mM sodium potassium tartrate was performed to exclude the effect of tartrate and potassium ions on the proton gradient. (D) Acr3p-mediated metalloid/H^+ exchange kinetics. The apparent K_m values (2 mM) for the As(III)/H^+ and Sb(III)/H^+ movement were estimated by Lineweaver–Burk plots. Inset, the rate of acridine absorbance increase versus metalloid concentration during initial time (60 s) after the addition of metalloids into the reaction media. (A–D) Presented values are representative for the results obtained in three to four independent experiments with each experiment done in triplicate.

Table 1
Accumulation of As(III) or Sb(III) into the yeast inside-out plasma membrane vesicles with the established proton gradient.

Metalloid concentration (mM)	Metalloid uptake (nmol mg^{-1} vesicle protein 3 min^{-1})[a]	
	*acr3*Δ + pACR3-GFP	*acr3*Δ + pUG35
As(III)		
2	175 ± 15	ND[b]
3	262 ± 31	ND
8	407 ± 28	ND
10	409 ± 31	11 ± 3
Sb(III)		
1	87 ± 7	ND
2	109 ± 3	ND
8	138 ± 5	ND
10	136 ± 7	4 ± 1

[a] All values were corrected for the amount of metalloids non-specifically bound to the plasma membranes measured in control conditions without the pH gradient (data not shown). The values presented are average with the SD of two replications.
[b] Not determined.

both lower level of Acr3 expression in the presence of Sb(III) than under As(III) stress [14] and slower rate of Sb(III) transport via Acr3p (this work) contribute to a minor role of Acr3p in mediating Sb(III) resistance in *S. cerevisiae* but not inability of Acr3p to recognize Sb(III) as a substrate. Considering the fact that, at the physiological pH, both arsenic and antimony are in the form of neutral $As(OH)_3$ and $Sb(OH)_3$ species [32], which are transported not only by an unrelated bacterial metalloid antiporter ArsB for the exchange with protons [18,33] but also by aquaglyceroporins for both uptake and efflux in various organisms [24,33–37], we favor the hypothesis that Acr3p actually mediates $As(OH)_3/H^+$ and $Sb(OH)_3/H^+$ antiport.

In addition to the Acr3 family found in prokaryotes and eukaryotes, a second family of metalloid efflux proteins called ArsB exists in bacteria [5]. The ArsB proteins possess the topology of 12 transmembrane spans and belong to the major facilitator superfamily of transporters [5,38]. Although there are no sequence or structural similarities between ArsB from *E. coli* and Acr3p from *S. cerevisiae*, both proteins appear to be trivalent metalloid/H^+ antiporters conferring resistance to As(III) and Sb(III) by transporting toxic metalloids out of the cells [14,39,40]. Considering the kinetic properties of

metalloid efflux (Fig. 2) as well as the results of the yeast growth tests in the presence of As(III) and Sb(III) (Fig. 1D), we may clearly conclude that Acr3p is a functional analog of bacterial ArsB despite the fact that both proteins belong to phylogenetically different families. The concentration dependence for As(III) and Sb(III) transport via ArsB and Acr3p exhibited saturation kinetics that could be fitted to the Michelis–Menten relationship, although the K_m and velocity of metalloid transport catalyzed by these antiporters are considerably different. The apparent K_m of 0.14 mM and 0.043 mM was calculated for ArsB-mediated As(III) and Sb(III) efflux, respectively, with the V_{max} values of approximately 182 nmol/mg protein per minute for Sb (III) and 2 nmol/mg protein per minute for As(III) [39,40]. It has been shown that, at 0.1 mM metalloid concentration, the rate of Sb(III) efflux via ArsB was 35-fold higher than that of the As(III) [39]. Conversely, the rate of Acr3p-mediated As(III) efflux was 3-fold higher than that of Sb(III) at 10 mM metalloid concentration (Table 1, Fig. 2D) and the apparent K_m of 1 mM was generated for the transport of both As(III) and Sb(III) (Fig. 2D). Thus, at the same concentration of metalloids, ArsB displays considerably higher capacity for Sb(III) efflux contrary to Acr3p, which is capable to transport 3-fold more As (III) than Sb(III). In addition, the above results show that the affinity of both proteins to metalloids is significantly different since ArsB-mediated transport was detectable at 0.05 mM and saturable at 1 mM concentration of metalloids [39,40], whereas Acr3p-mediated efflux started at 1 mM and saturated at 5 mM As(III) and 8 mM Sb(III).

Although the kinetic properties and the rates of metalloid transport via ArsB and Acr3p are quite different, both proteins are capable of efflux-mediated detoxification of bacterial and yeast cells from As (III) and Sb(III) by the same metalloid/proton antiport mechanism. Thus, the question remains why these two distinct systems of metalloid efflux have evolved in prokaryotes and why the *arsB* gene has been lost in eukaryotes.

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